Addendum

Background:

After submitting our review report on Corman *et al.* (referred hereinafter as CD-report) and republishing it on a scientific preprint server [50] and Researchgate.net [51] we offered the report for public discussion at cormandrostenreview.com on 27th November 2020. The scientific community provided additional literature, references, and analyses concerning the CD-report and the Corman *et al*. manuscript. Several "advocatus diaboli" confronted us with correct or assumed problems in our report. The most common critique of the CD-report was the lack of "wet lab" experiments to support our concerns over the technical flaws in the PCR protocol.

Aim:

This vibrant debate on our CD report has provided additional information worthy of further public documentation to address these critiques. We summarize the current published knowledge of "wet lab testing", routine diagnostic use and validation of the original PCR-Protocol described by Corman *et al.* Further, this addendum highlights that independent research groups (some of them with Corman and/or Drosten as author) also pointed out important concerns with the original manuscript and Corman PCR protocol distributed by the WHO. Many of these references were already provided by the authors of the original CD-report but it is worth underscoring their relevance to the formation of our critiques of the CD manuscript.

Methods:

We searched the literature for 'SARS-CoV-2 qPCR' and 'Corman' or 'Charité'. Then we combined these references with those provided by other scientists working in relevant Life Sciences/data analysis fields.

In the first section of the addendum, the publications will be discussed point by point, highlighting their findings in relation to the CD-report. In a second section, additional aspects about the Corman *et al*. publication are discussed. This spans a meta-analysis of the unusual peer-review process, timeframes, and further technical vulnerabilities of the Corman *et al*. PCR-protocol.

An additional concern was raised about the CD-report regarding the discussion of appropriate controls. We cite several studies that underscore the importance of internal controls in assessing viral load and the lack of such internal controls in the Corman qPCR method. These internal controls are required for normalizing swab sampling variance and

they are critical for interpreting viral load. They are notably absent from the Corman PCR protocol. Several people also expressed confusion regarding the NCBI submissions provided by Corman *et al*. The sequences provided lack two of the target gene sequences Corman *et al.* claim to target. The only sequences referenced in the manuscript are listed (KC633203, KC633204, KC633201, GU190221, GU190222, GU190223) and none of these have sequences that match their N and E gene primers. This not only brings their validation into question but also prevents others from reproducing the work presented in Corman *et al.*

Results:

We present 20 scientific publications providing ‘wet lab’ evidence of the performance of the Corman *et al.* PCR protocol. Of those, 17 found problems with incorrect primer design (mismatches, dimer formation, melting temperature) in the SARS-CoV-2 specific “confirmatory” test named RdRp-PCR for “RNA-dependent RNA-polymerase” or the E-gene assay.

These documented problems include:

- Documented primer dimers and False Positives in non-template controls (NTCs)
- Documented poor sensitivity and False Negatives compared to other assays
- No internal control to normalize the sample preparation variability and its impact on viral load estimation
- No defined Ct for calling samples “Positive cases”
- Poorly documented positive controls and sequences used in their study

Conclusion:

We believe the references provided in this addendum itemize the scientific consensus evident in the literature regarding the flaws in the original PCR detection method for SARs-CoV-2 published by Corman *et al.*. Further, since several important flaws were published in peer-reviewed journals, the lack of correction of the original PCR protocol by either Eurosurveillance or as an update in the Charité-WHO protocol brings into question the scientific integrity of the authors of Corman *et al.* These references settle any remaining debate that the Corman *et al.* manuscript should be retracted on technical grounds alone. The rapidity of the peer-review and conflicts of interest are even more troubling.

Addendum: Peer reviewed literature and preprints covering wet experiments, *in silico* analysis of the Corman Drosten protocol-design, meta-data analysis on EuroSurveillance.org and further discussion

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Note: sentences written in italics are original citations from the respective publications

Section 1:
A. Wet lab evidence of primer design flaws

The primer pair for the RdRp gene was shown to create a positive PCR test result in the absence of SARS-CoV-2. This can happen when the primer design is suboptimal and the primers react with themselves in the absence of the virus. Insufficient test specificity and primer design flaws seen in Corman-Drosten's SARS-CoV2 qPCR assay creates a high number of false positive and false negative results.

a. Background and Pinollel et al. (Letter to the editor of Eurosurveillance)

We have listed 20 references that give compelling wet-lab evidence for flaws in primer design and methodological validation of the PCR testing protocol by Corman *et al*. These studies nullify the most common complaint voiced (no wet-lab evidence) regarding the retraction letter.

There is no need for the authors of the Corman-Drosten (CD manuscript) retraction request to perform wet-lab experiments to prove these deficiencies as those experiments are already evident in fully peer-reviewed articles. These papers represent diverse labs with diverse authors and different jurisdictional influences on the scientific funding and research.

Initially, it is important to underscore the other complaint already evident with the CD manuscript.

Pillonel et al. - Letter to the editor: SARS-CoV-2 detection by real-time RT-PCR [16]:

“After careful review of the initial manuscript and analysis of SARS-CoV-2 and other coronavirus sequences, it appeared that the proposed RdRp reverse primer contained an incorrect degenerate base (S), that does not match with the SARS-CoV-2 RNA sequence, as shown in the alignment of Corman et al. Figure 2.”
[...]
“These observations based on in silico alignments should be confirmed by wet-laboratory experiments, but they could explain the lower sensitivity of the RdRp RT-PCR also shown by Vogels et al. and point towards potential improvements.”

“As the pandemic spreads, many laboratories worldwide, including in low-resource countries that may not rely on expensive commercial kits, implement routine

diagnostic tests. Thus, we think that such information is critical to ensure a proper detection of SARS-CoV-2 infections, allowing efficient isolation and preventing further transmission of the virus."

Corman et. al: Authors' response: SARS-CoV-2 detection by real-time RT-PCR [31]:

"Our strategy during establishment was to use a synthetic target for the SARS-CoV-2 E gene assay, while validating amplification of a full virus genome RNA using the RdRp assay that is specific for both, SARS-CoV and SARS-CoV-2, with the latter not being available to us in the form of an isolate or clinical sample at the time. Based on experimental validation, it later turned out that the mismatched base pairs do not reduce RT-PCR sensitivity and are not to be seen as the reason for somewhat higher Ct values with the RdRp assay as compared to the E gene assay."

Since Nalla *et al.* is cited in this author's response as reference, also see section ***16. Nalla et al.*** in this Addendum.

This Addendum challenges the authors' response (Corman *et al*.) and claims to Pillonel *et al*.'s letter to the editor (Table 1).

Table 1: Main findings in the publications reviewed

Publication	**Proof of false positive (FP) or low sensitivity (LS)**	**Discussion of high CT**	**Detected mismatches**	**Primer dimers**	**Authors modified primers**	**Reason**
Muenchhoff et al	RdRp (LS)	E-gene (≥37) RdRp gene (≥40)	In RdRp reverse		RdRp reverse	high difference in melting temperature
Jung et al	RdRp (FP)					
Etievant et al	E-gene (FP) RdRp gene (LS)			Detected with primer contamination		
Gand et al	N-Gene (LS)		N-gene forward and reverse RdRp reverse RdRp probe			Mentions WHO needs to update Corman errors
Konrad et al	E-Gene (FP)	E-gene, FP ≥ 35		Discussed for E-gene		
Sethuraman et al						Only review
Nalla et al	N-gene (LS) RdRp-gene (LS)					
Vogels et al	RdRp-gene (LS)	For N gene of CDC only	RdRp-gene			
Kuchinski et al						
Ratcliff et al			RdRp			Correcting the mismatch
Jaeger et al				Dimer formation with Taqman or fluorogenic probes detected		CDC primers only
Khan et al			RdRp reverse (T)			In silico
Opota et al	E-Gene (FP+FN)					
Barra et al						Higher primer concentration in order to improve detection limit
Santos et al			RdRp reverse (T)			
Anantharajah et al	RdRp (LS)		RdRp reverse (T)			
Nalla et al	RdRp (LS) E gene (LS)					
Dahdouh et al		10-16 Ct variance in Sample prep. Requires human amplicon to normalize				Critical to have Internal controls
Poljak et al						Critical to have Internal controls
Boutin et al	15% disagreement					Critical to have Internal controls
Pfefferle et al					Modified primers to prevent primer dimers	

b. Review of the literature

1. Muenchhoff *et al.*

Muenchhoff *et al.* compare seven different labs using various PCR protocols including the primers described in the CD manuscript. Six out of seven laboratories in the Muenchhoff *et al*. paper [1] tested the original primer pairs described in the Corman-Drosten paper. Muenchhoff *et al*. also refers to the official WHO-recommendation of the protocol [2].

According to table 1 in the Muenchhoff *et al*. paper, the Corman-Drosten protocol components (primers, gene assays, etc.) are labeled and referred to as “Charité genes” and TIB-Molbiol is listed as the manufacturer of the corresponding primers/probes.

As a proficiency test for inter-laboratory performance evaluation, a series of 10-fold dilutions of one of the SARS-CoV-2 PCR positive RNA samples was sent out to all seven laboratories. As a result, 5 of 6 laboratories were able to find as low as 5 copies of SARS-CoV-2 RNA by Charité E-gene PCR, and all 50 or fewer copies by the Charité RdRp gene PCR. The three labs amplifying the Charité N-gene PCR managed to detect 5 of the spiked RNA molecules.

In parallel to the intra-laboratory testing of the RNA dilution series, the main authors of the manuscript compared the sensitivity of different primer pairs with a digital droplet PCR in their laboratory (Laboratory 1).

Based on the digital droplet PCR, the authors concluded that the “Charité E gene” primer pair performance is comparable with the “CDC N primer pairs”; both show similar sensitivity, but the N gene and the RdRp gene assays are **significantly less sensitive** with the positive RNA samples tested (Figure 1).

Figure 1, taken from Muenchhoff *et al*.: Digital droplet PCR quantification of the distributed dilution series of nucleic acid eluate of SARS-CoV-2-positive clinical material, Germany, March 2020.

In addition, a test of 28 samples derived from pre-tested CDC N1-gene positive patient-samples in Laboratory 1 revealed that all Charité primer pairs showed a Ct with a median of around 37 (CDC N1 and Charite' E) and 40 or higher (Charite' RdRp) and a "modified" improved Charite' RdRp showed a Ct of 36 as median (Figure 2). None of the patients' samples were positive at a Ct of 32 or lower.

Figure 2 taken from Muenchhoff *et al. (Figure 3)*:
RT-PCR results of respiratory samples with low positivity, SARS-CoV-2 detection, Germany, March 2020 (n = 28 samples). The Charité RdRp assay is the worst performing.

CDC: Centers for Disease Control and Prevention; Cp: crossing point; E: envelope gene; N: nucleocapsid gene; RdRp: RNA-dependent RNA-polymerase gene; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Nucleic acid eluates of clinical respiratory specimens that initially showed low positive results in the CDC *N1* reaction (Cp value > 35) were retested side by side in the CDC *N1*, the Charité *E* and original *RdRp* reaction and using the modified *RdRp* primers on the Roche LightCycler 480 using the QuantiNova Multiplex RT-PCR kit. Cp values are shown with positive amplifications beyond cycle 40 shown as > 40 (dotted line).

This modified reverse RdRp primer was created by the authors due to a mismatch of one of the bases in the original Charité primer to the reference sequence Wuhan-Hu-1/2019, which was replaced by the correct "T" and the selection of another "T" in a second position, where the original Charité primer had an ambiguity base (C or T) which should be a T. Further, the Muenchhoff *et al.* authors claimed that:

"Based on computation using Primer Express v3.0 (Applied Biosystems, Dreieich, Germany) annealing temperatures were predicted to be 64 °C for the RdRp forward and 51 °C for the RdRp reverse primer of the Charité protocol. This temperature difference may result in reduced PCR efficiency" [1]

Both primer sequences were shown in their supplemental figure S1 (note: the reverse primer is given as a complementary sequence). (Figure 3)

Figure 3 taken from Muenchhoff *et al*. (figure S1):
The forward primer and the reverse complement of the reverse primer of the RdRp reaction from the Charité protocol is aligned to the reference sequence Wuhan-Hu-1/2019 (NCBI NC_045512.2). The red box indicates an ambiguity base S, i.e. G or C, at a position where T should be the reverse complement. The black box indicates an ambiguity base Y, i.e. T or C, at a position where T would exist, and the green box indicates an R where A can be used based on currently available sequence data.

The modified RdRp primer pair now has the correct melting temperature, however the modified reverse primer is now unusually 30 bp long. (Table 1)

Table 1: Modified RdRp primer pair, Length, Tm, GC% - values - values according to Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)

RdRp	**Sequence (5' -> 3')**	Length	Tm	GC%	Self complementarity
Forward primer	AAATGGTCATGTGTGGCGGT	20	60.54	50.00	4.00
Reverse primer	GTTAAAAACACTATTAGCATAAGCAGTTG A	30	59.53	30.00	5.00

This need for primer modification is a direct result of the authors of the Corman *et al.* protocol skipping mandatory and simple-to-test primer design QC steps. Screening for primer dimers or hairpins is a crucial step to avoid false positive as well as false negative

results. Open-source software such as the web tool by Thermofisher [3] is freely available on the internet to perform this critical screening and is shown below this review of the Muenchhoff *et al*. section. (Figure 5)

Conclusion Muenchhoff *et al.*

The rapid communication-publication (also published in Eurosurveillance) concludes that the RdRp assay in the Corman-Drosten paper is deficient and needs to be replaced. The paper demonstrates sensitivity issues, which would support false negatives being generated by the test.

"A reduced sensitivity was noted for the original Charité RdRp gene confirmatory protocol, which may have impacted the confirmation of some COVID-19 cases in the early weeks of the pandemic. The protocol needs to be amended to improve the sensitivity of the RdRp reaction." [1]

Further discussion of Muenchhoff *et al.*

1. The fact that the Corman *et al.* primers were given to testing companies (Labor Berlin, Tib Molbiol) and commercially sold as Light Mix diagnostic Test kits (LightMix® Modular SARS-CoV / COVID19, RdRp / LightMix®Modular SARS-CoV / COVID19, E-gene, TIB Molbiol, Roché diagnostics) and cemented into WHO guidelines prior to peer-review should concern everyone. This is 'science by press-release' where authoritative bodies (the WHO) are used to advertise a manuscript before it has seen proper peer-review. After the PCR protocol is pushed through the WHO, we additionally see a rushed 24 hour peer-review, while furthermore the authors being on the editorial board of the journal (Eurosurveillence) performing the review. This is a dangerous practice when undisclosed conflicts of interest (COIs) exist. It is now known to have produced erroneous results and contributed to global lockdowns.
2. The author's urgency in communication with the WHO, is not replicated in addressing the errors in Muenchhoff *et al.* which Drosten is an author of. These known errors were published on June 18th 2020 and yet the WHO primers are not updated as of today! Why the race to get these primers to testing companies and onto the WHO website in January 2020? Why the lack of urgency in addressing the false negatives (FNs) and false positives (FPs) 6 months after publishing Muenchhoff? Testing labs generate more revenue with higher positivity tests due to contact trace testing. This COI may explain the different urgency?
3. Christian Drosten is co-author of the Muenchoff *et al*. publication, which was released on 18th June 2020 at Eurosurveillance (Figure 4). The study clearly

concludes that the Corman-Drosten paper RdRp primer designs must be exchanged and/or removed from the protocol due to sensitivity issues. Other papers provided below highlight water samples (NTCs) amplifying. Thus, the protocol lacks sensitivity for the RNA target and specificity in the signal it provides. It produces both FPs and FNs.

4. In the Muenchhoff *et al.* publication Christian Drosten does not properly disclose his COIs and affiliations (Figure 4). As in the Corman-Drosten paper, his affiliation as Director of Virology at Labor Berlin is not listed, a laboratory which operates commercially within the PCR-testing realm. [5]

Figure 4: Christian Drosten fails to list his affiliations properly: He is Director of Virology at Labor Berlin, a commercially oriented company which offers PCR-testing.

Rapid communication

Open Access

Multicentre comparison of quantitative PCR-based assays to detect SARS-CoV-2, Germany, March 2020

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Figure 5: Corman-Drosten protocol RdRp probe, hairpins and homodimers.

```
Self-Dimers:

1 dimer for: Corman_RdRp_SARSr-P2.a
5-ccaggtggaacatcatcaggtgatgc->
            ||||| | | |||||
        <-cgtagtggactactacaaggtggacc-5
```

RdRp gene primers also have a homology to the E-gene primers, which was already discussed in the main review report [4], see Figure 6.

Figure 6: A test with Thermo Fisher's primer dimer web tool reveals that the RdRp forward primer has a 6bp 3`prime homology with Sarbeco E Reverse.

```
Cross Primer Dimers:

Corman_RdRp_SARs_F1 with Corman_E_Sarbeco_R
Corman_RdRp_SARs_F1
5-gtgaaatggtcatgtgtggcgg->
             ||||||
           <-acacacgcatgacgacgttata-5

Corman_RdRp_SARs_F2 with Corman_E_Sarbeco_R
Corman_RdRp_SARs_F2
5-gtgagatggtcatgtgtggcgg->
             ||||||
           <-acacacgcatgacgacgttata-5
```

While most labs run these tests in different wells (1-plex), it is certainly risky practice to have primer dimers between 1-plexes, especially when factoring in that liquid handling of millions of tests can create numerous contaminations. Such primer contaminations are not just a theoretical risk but are in fact reported in the peer-reviewed literature referred to below.

2. Jung *et al.*

The authors tested several PCR primer pairs for amplification of isolated N from SARS-CoV-2 infected cell cultures. As a result Jung *et al*. did not recommend the Corman *et al.* RdRp PCR (named Charite PCR in the publication) for diagnostic purposes.

Jung *et al*. clearly refute a commonly voiced misconception, that reduced sensitivity of the Corman *et al.* protocol could only manifest itself with false negatives and should not create false positives.

"Unexpected amplifications from NTC samples were observed with the RdRp_SARSr (Charité) set. The electrophoresis and melting curve analysis showed non-specific amplification at lower positions (Lane 5, Figure S5b) and temperatures (Figure S5a)." [7] (Figure 7)

Jung *et al.* further demonstrate these primers have reduced sensitivity as reported by Muenchhoff *et al.* False negatives and false positives are generated with the Corman-Drosten primer design.

Promiscuous primers not only fail to amplify targets in some samples, they also amplify non-specific sequences in other samples which they should not amplify. **In this case they amplify water** (NTC). The authors demonstrate the Charité RdRp PCR generate positive water signals but to a lesser extent than the US and China CDC primer combinations (see * in lines 1,3 and 5 in Figure 6b). However, primer dimer formation is seen in the gel image with the US CDC (line 1) and the Charite RdRp (line 5) primer pair (arrow), (see modified Figure 7).

Figure 7 taken from Jung et al. (Figure S5.):
(a) Melting curve analysis and (b) polyacrylamide gel image of PCR products with primer- probe sets that show positive signals in the NTC samples. M: DNA ladder; 1: NTC sample with 2019-nCoV_N1 (US CDC); 2: PCR product with 2019-nCoV_N1 (US CDC); 3: NTC sample with N (China CDC); 4: PCR product with N (China CDC); 5: NTC sample with RdRp_SARSr (Charité); 6: PCR product with RdRp_SARSr (Charité)

Conclusion:

The RdRp PCR from the Corman *et al.* publication produces less false positive amplification than the US and China CDC N1 and N PCR, however it still produces a very problematic amplification of “water only” which is a clear no-go for a PCR reaction intended for diagnostic use.

3. Etievant *et al.*

This citation also demonstrates poor results with the Charité E gene-assay and attributes this to primer contamination and primer dimers. Etievant *et al*. highlights the dimerization that can occur between E and RdRp gene-assays:

"The E Charité and N2 US CDC assays were positive for all specimens, including negative samples and negative controls (water). These false-positive results were explored (details below), but the sensitivity of these assays was not further assessed." [8]

In theory, this should be a rare occurrence if labs are running singleplex assays without primer contamination, yet it is readily found in peer-reviewed literature with these exact assays and conditions by Etievant *et al*. Even with singleplex assays free of primer contamination, RdRp probe forms a hairpin and a self-dimer and this likely explains the reduced sensitivity of this assay (Figure 8).

The Etievant *et al.* study demonstrates that the CT values are in question as the Corman-Drosten paper did not disclose this important detail:

"*It is worth noting that the Charité assay was the first to be published at the early stage of the pandemic and has been widely used worldwide."*
[...]
*"Of note, we did not apply the Ct cut-off values above, in which a sample would be considered negative, **since such values were not provided** in the protocols made available by the referral laboratories."*
[...]
*"As previously reported, we identified probable **primer contamination** using N2 US CDC and E Charité, which prevented us from further evaluating their sensitivity and specificity." [8]*

These authors could not determine the sensitivity and specificity of these assays due to the flaws we explain in the retraction request. Known sensitivity and specificity are paramount to clinical diagnostics as described in Klement & Bandyopadhyay [9].

Figure 8 taken from Etievant *et al. (Figure 1)*:
Mean Ct values and standard deviations obtained using five PCR-based methods for SARSCoV-2 detection. Serial dilutions of SARS-CoV-2 cell culture supernatants were used and are represented by a single color (10^{-5} blue, 10^{-6} red, 10^{-7} pink, 10^{-8} green). A point in the ND (non-detected) column (Ct value axis) indicates a negative result for one replicate.

Upon exploration of the false positive signals obtained with the Corman *et al.* E-gene, the authors noted:

“For E Charité, negative samples showed two amplicons, one at 84 base pairs (bp) and one at 121 bp, whereas the positive sample only had one amplicon at 121 bp, which is close to the expected size of a specific amplification (Table 1). Thus, the false-positive amplification obtained using E Charité might be derived from a contamination (amplicon size at 121 bp) but could also be associated with an aspecific amplification (amplicon size at 84 bp).” [8] (Figure 9)

Figure 9 taken from Etiviant et al. (Table 1): Charité assay targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

Conclusion:

The Corman *et al.* E-primer pair produces false negatives either due to contamination or to unspecific amplification.

4. Gand *et al.*

Gand *et al*. [10] notes that the Charité primers were the most widely used in Europe in the spring of 2020, referencing Reusken *et al*. published at the end of January 2020 at Eurosurveillance:

"*The RT-qPCR test developed by Corman and colleagues at Charité (Berlin) is the most widely used in Europe.*"

A publication by Chantal Reusken and Marion Koopmans is referenced [52] (Figure 10). Both are co-authors of the Corman-Drosten-paper. Chantal Reusken is also on the editorial board of Eurosurveillance.

Further global use rates of the CD assays in the time frame January to December 2020 are not known and are difficult to deduce from the scientific literature. Since Charité Berlin did not claim any patent ownership for the invention, it is difficult to track usage with traditional royalty streams or estimates of revenues [11].

Figure 10 taken from Reusken *et al.* (Figure 2):
Status of availability of molecular diagnostics for novel coronavirus (2019-nCoV) in EU/EEA countries as at 29 January 2020 (n = 46 laboratories)[a]

As highlighted in our initial review, the authors (Gand *et al.*) mention that the false positives observed were predictable by in-silico analysis.

"The sensitivity of Assay_2_RdRp-P2 (Charité) was already demonstrated in the wet lab to be lower than that of other assays investigated in this study, and it was hypothesized that these SNPs present in almost all SARS-CoV-2 genomes could be the reason for this. As the utmost sensitivity is required for SARS-CoV-2 detection, especially when the viral load is low depending on the time and nature of the sampling, it might be proposed to correct such mismatches with the aim to potentially increase the sensitivity of Assay_2_RdRp-P1, Assay_2_RdRp-P2, Assay_8_RdRp, and Assay_10_E. The SNP present in the reverse primer of Assay_5_N was already corrected in a revised version of the protocol ***but has not yet been updated in the WHO*** *technical guidance."*

The authors point out that similar false positive results were predictable with their in-silico analysis and that the WHO has yet to address the errors in the Drosten primers.

*"Interestingly, for Assay_2_RdRp-P2, similar false-positive results **as obtained in our in silico study** were obtained in the wet lab by Chan and colleagues, who detected SARS-CoV when using the probe P2 targeting the RdRp gene that is considered strictly specific to SARS-CoV-2. This indicates that our **in silico analysis** can be backed up by in vitro data."*

5. Konrad *et al.*

Konrad *et al.* report similar problems with false positive (FP) signals at high Ct. They report 61% FPs with their first test system. They improve upon this by changing their PCR master mix but still achieve a 5.1% FP rate with the improvement.

*"We found that the SARS-CoV E gene screening assay with the QuantiTect Virus +Rox Vial kit showed moderate to high amounts of unspecific signals in **late cycles in 61%** (451/743) of the tested patient samples and also of negative extraction and non-template controls (Table, Figure 2), which complicated the evaluation of the qPCR result. The RdRp assays were basically free from such unspecific signals in late cycles."* [12] (Figure 11, Figure 12)

Figure 11: taken from Konrad et al (Figure 2):
Example image of real-time RT-PCR curves of the gene assay with unspecific signals at late cycles, Bavaria, February 2020

Amplification

RFU

10^4

10^3

10^2

0 10 20 30 37 40

Cycles

Negative patients sample producing positive signal at CT over 37

RFU: relative fluorescence units.
Curves: 1: Wuhan coronavirus 2019 E gene positive control; 2: SARS-CoV Frankfurt 1 RNA positive control; 3,4,6,8: negative patient samples; 5: extraction negative control; 7: non-template negative control.

Signal is given in log scale with threshold = 200. PCR was performed with SuperScript III system and E gene primers and probe as published in [5]. Curves of positive controls (1 and 2) show expected sigmoid curves. Curves 3–6 show unspecific signals with increase above threshold. Curves below threshold were not considered as significant signals (7 and 8).

Figure 12 taken from Konrad et al.*(Table)*:
Comparison of two different one-step real-time RT-PCR systems with SARS-CoV-2 assays from Corman et al. [5] and a commercial test kit with kit-specific assays, Bavaria, February 2020

Real-time RT-PCR system	PCR efficiency (%)[a], linearity (R^2)	Limit of detection (copies/reaction)	Unspecific signals count in E gene assay in total[b]	Unspecific signals in E gene assay (%)[b]	Run time (hours)
QuantiTect Virus+Rox Vial kit (QIAGEN)	ND	ND	451/743 (75/126 NC, 376/617 patient samples)	60.7	1:50
SuperScript III One-step RT-PCR System with Platinum TaqDNA Polymerase (Invitrogen)	95 / 0,99[c]	50[c]	13/257 (2/38 NC, 11/219 patient samples)	5.1	1:28
RealStar SARS-CoV-2 RT-PCR kit 1.0 (Altona)	125 / 0,97[d]	10[d]	0/111 (0/38 NC, 0/73 patients samples)	0	2:15

NC: negative control samples; ND: not determined.
[a] $E = 10^{-1/slope} - 1$.
[b] Indicated counts and percentage values of unspecific background signals in the SARS-CoV E gene assay are based on the total number of tested patient samples as well as the negative extraction and non-template controls.
[c] Only for RdRp gene assays, tested with four replicates of SARS-CoV Frankfurt 1 RNA [6]; 10-fold serial dilutions were determined. For the E gene, the assay was not linear.
[d] Only for the E gene, tested with two replicates of synthetic Wuhan coronavirus 2019 E gene control and SARS-CoV Frankfurt 1 RNA each [6]; 10-fold serial dilutions were determined.

The authors conclude this is due to nonspecific signals from dimerisation of primers and probes as mentioned in our retraction request:

*"Using commercial kits with optimised target regions and primer sequences (in the E gene and SARS-CoV-2-specific S gene) ruled out the unspecific signals completely. Hence, reasons for the observed **unspecific signals may be dimerisation of primers** and probes and/or unspecific primer binding and polymerase activity in the targeted region of the E gene, probably also depending on thermal profile and cycler-specific differences, or most likely a combination of these factors."* [12]

6. Sethuraman *et al.*

Sethuraman *et al.* did not perform experiments themselves but instead refer to Nalla *et al.* in connection with the problematic Charité primers. They attribute this to the mismatch in the reverse primer:

"The sensitivities of the tests to individual genes are comparable according to comparison studies except the RdRp-SARSr (Charité) primer probe, which has a slightly lower sensitivity likely due to a mismatch in the reverse primer." [13]

7. Nalla *et al.*

Nalla *et al*. performed sensitivity tests with the three original Corman *et al.* PCR compared to the US CDC N genes and the RdRp of their own lab. Here, the E-gene test was very sensitive and the N-and RdRp gene PCRs showed reduced sensitivity compared to others.

"Assays using UW RdRp and Corman N-gene primer-probe sets have limits of detection (LODs) of about 790 viral genomic equivalents per reaction."
[...]
"*Assays using the Corman RdRp and E-gene sets were found to have LODs of about 316 viral genomic equivalents per reaction."*
[...]
"*Assays using the CDC N2 and Corman E-gene primer-probe sets were more sensitive than those using the CDC N1 and Corman RdRp sets and the BGI kit."* [24], (Figure 13)

Figure 13: Table reproduced from Nalla *et al.*

TABLE 2 Relative performance of SARS-CoV-2 detection assays using five different primer-probe sets[a]

Sample ID	CDC N1	CDC N2	CDC N3	Corman RdRp	Corman E-gene
SC5777	24.5	23.2	23.3	29.0	24.9
SC5778	30.2	30.6	30.1	34.8	31.9
SC5779	33.3	32.8	32.0	36.5	34.7
SC5780	14.6	13.7	13.9	19.2	15.1
SC5781	15.1	14.1	14.3	20.2	16.2
SC5782	21.8	20.9	21.0	26.9	22.6
SC5783	16.0	14.9	15.6	20.8	16.9
SC5784	36.0	35.6	Negative	Negative	35.4
SC5785	27.8	27.3	27.4	32.7	28.9
SC5786	23.9	24.0	24.3	29.4	25.6

[a]Cycle thresholds are displayed.

The Nalla *et al.* authors include a panel of other respiratory viruses in their PCR testing, however, results are mentioned for the CDC N1 and N2-primer probe sets only, not for the Corman *et al.* primer/probes combinations, despite a sentence in the discussion claiming:

"Of the seven different primer-probe sets and one testing kit that we evaluated, all were found to be highly specific with no false-positive results observed when assays were run on samples positive for a number of other respiratory viruses." [24]

8. Vogels *et al.*

Vogels *et al.* describe the errors in the RdRp-SARSr_R Charité primer with 99.8% mismatch frequency in SARs-CoV-2. This is due to the Corman-Drosten primer design being performed and verified on the basis of a non-relevant SARs-CoV-1 sample (Figure 14, Figure 15):

"Thus far, we detected 12 primer–probe nucleotide mismatches that had occurred in at least two of the 992 SARS-CoV-2 genomes. The most potentially problematic mismatch is in the RdRp-SARSr reverse primer, which probably explains the sensitivity issues with this set. Oddly, the mismatch is not derived from a new variant that has arisen, but rather that the primer contains a degenerate nucleotide (S, binds with G or C) at position 12, and 990 of the 992 SARS-CoV-2 genomes encode for a T at this genome position." [14]

Figure 14 taken from Vogels *et al*. (Table 2):
High-frequency primer and probe mismatches may result in decreased sensitivity for SARS-CoV-2 detectionTable

Table 2 | High-frequency primer and probe mismatches may result in decreased sensitivity for SARS-CoV-2 detection

Institute	Primer–probe	Primer–probe position 5′–3′	Genome position 5′–3′	Primer–probe nucleotide	Nucleotide in ref. genome[a] (RC)	Expected target nucleotide	Mismatch target in genomes[b] (frequency)
China CDC	CCDC-N-F	1	28,881	G	G (C)	C	T^{RC} (126/992; 12.7%)
	CCDC-N-F	2	28,882	G	G (C)	C	T^{RC} (126/992; 12.7%)
	CCDC-N-F	3	28,883	G	G (C)	C	G^{RC} (126/992; 12.7%)
	CCDC-ORF1-F	17	13,358	C	C (G)	G	A^{RC} (2/992; 0.2%)
	CCDC-ORF1-P	26	13,402	T	T (A)	A	C^{RC} (4/992; 0.4%)
Charité	E_Sarbeco_R	12	26,370	G	C (G)	C	T (4/992; 0.4%)
	RdRp-SARSr_R	12	15,519	S	T (A)	C or G	**T (990/992; 99.8%)**
HKU	HKU-N-F	4	29,148	T	T (A)	A	G^{RC} (5/992; 0.5%)
US CDC	2019-nCoV_N1-P	3	28,311	C	C (G)	G	A^{RC} (2/992; 0.2%)
	2019-nCoV_N1-R	15	28,344	G	C (G)	C	A (4/992; 0.4%)
	2019-nCoV_N3-F	8	28,688	T	T (A)	A	G^{RC} (39/992; 3.9%)
	2019-nCoV_N3-R	14	28,739	C	G (C)	G	T (4/992; 0.4%)

[a] Nucleotide (DNA form) found in the reference genome (NC_045512) and its reverse complement (RC). [b] Mismatch target is the disagreement between the expected target nucleotide and the nucleotide in the genome. Listed are mismatched nucleotides with primers and probes with frequency >0.1% in 992 genomes inspected in this analysis. The column at the far right highlights the various frequencies of mismatches, which would represent a mispairing following binding of the primers listed above. The high-frequency mismatch in the RdRp-SARSr reverse primer is highlighted in bold. A list of degenerate nucleotides incorporated into the primer and probe sequences can be found in Supplementary Table 4. Data used to make this table can be found in Source Data Fig. 4.

Vogels *et al.* further states:

“At 10^{0} and 10^{1} viral RNA copies µl–1, our results show that all primer–probe sets, except RdRp-SARSr and 2019-nCoV_N2, were able to partially detect (Ct < 40) SARS-CoV-2 from clinical sample.” (Figure 15, Figure 16)

Figure 15 taken from Vogels *et al*. (Fig.1):

Analytical efficiency and sensitivity of the nine primer–probe sets used in SARS-CoV-2 RT–qPCR assays.
a,b, Mean Ct values for nine primer–probe sets and a human control primer–probe set targeting the human RNase P gene tested for two technical replicates with tenfold dilutions of full-length SARS-CoV-2 RNA (a) and pre-COVID-19 nasopharyngeal swabs spiked with known concentrations of SARS-CoV-2 RNA (SARS-CoV-2 RNA-spiked mocks (b)). The CDC human RNase P (RP) assay was included as an extraction control. c,d, From the dilution curves in a,b, PCR efficiency (c) and y-intercept Ct values (measured analytical sensitivity) (d) were calculated for each of nine primer–probe sets. Symbols depict sample type: squares represent tests with SARS-CoV-2 RNA and diamonds represent SARS-CoV-2 RNA-spiked mock samples. Colours denote the nine tested primer–probe sets. Dashed lines indicate 90% PCR efficiency (c) and the detection limit (d).

Figure 16 taken from Vogels *et al*. (Fig.2):

Comparison of analytical sensitivity of SARS-CoV-2 primer–probe sets using pre-COVID-19 nasopharyngeal swabs.

The lower detection limit of nine primer–probe sets, as well as the human RNase P control from RNA extracted from nasopharyngeal swabs collected in 2017 spiked with known concentrations of SARS-CoV-2 RNA. Each primer–probe set was performed using 24 technical replicates of pooled-swab RNA without spiking SARS-CoV-2 RNA ('No virus'; six replicates with four independent pools each of four swabs) and eight replicates (two replicates with four independent pools each of four swabs) spiked with 100–102 viral RNA copies µl–1 of SARS-CoV-2 RNA. ND, not detected. Solid lines indicate the median and dashed lines indicate the detection limit.

9. Kuchinski *et al.*

Kuchinski *et al.* [15] also demonstrate the errors in the RdRp assay, with 99.6% samples having a mismatch sequence as described in Vogels *et al*. [14], (Figure 17). This was also raised by Pillonel *et al*. [16] and this particular correspondence letter can be found now attached to the Corman-Drosten manuscript as an erratum at Eurosurveillance.

Figure 17 reproduced from Kuchinski *et al*. (Table 2):
Frequency of mismatches between 15,001 SARS-CoV-2 genome sequences and 15 sets of oligonucleotides from early lab developed tests. The Charite group - RdRP is shown here only.

Assay	0 mismatches	1 mismatches	2 mismatches	3+ mismatches
Charité group - N	98,9%	0.9%	0.0%	0.1%
Charité group - RdRP	0.0%	99.6%	0.3%	0.0%
Charité group - E	99,6%	0,2%	0,1%	0,0%

Under section 3.3 it is stated:

*“**Pervasive single nucleotide mismatches in assays from Charité Group and Japan NIID:** Two sets of oligonucleotides had mismatches against all 15,001 SARS-CoV-2 reference genomes in our dataset: the Charité group’s RdRP gene assay and the Japan NIID’s N gene assay.”*

10. Ratcliff *et al.* PrePrint

Ratcliff *et al*. is still in PrePrint form but also explains the underperforming primer sequences circulated by the WHO and recommended by the Corman Drosten protocol.

*“Unexpectedly, the performances varied substantially depending on the detection method and target assayed, underpinning the need for in-house validation and optimization. The result also **challenges the notion that Ct values presented without context could be an informative metric for the progression of disease** and can be compared across different*

amplification techniques and laboratories."
[...]
"The Charité RT-PCR was based upon previously described primer/probes for the RdRP gene but with modifications to the antisense primer to ensure complete sequence complementarity with SARS-CoV-2 sequences."
[...]
"All primers and probes for the Charité and CDC N1 PCRs were obtained from ATDBio. All primer sequences and working concentrations are available in Table 1." [17] (Figure 18)

Figure 18 taken from Ratcliff *et al.* (Table 1):
Primer and Probe Sequences for Nested PCR and RT-qPCR

PCR Assay	Primer Name	Sequence	Reaction concentration
Nested PCR	nF1	AYTCAATGAGTTATGAGGAYCAAGATGC	400 nM
	nR1	GACATCAGCATACTCCTGATTWGGATG	400 nM
	nF2	TAGTACTATGACMAATAGACAGTTYCATC	500 nM
	nR2	CCTTTAGTAAGGTCAGTCTCAGTCC	500 nM
Charité RdRP	RdRp_SARSr F	GTGARATGGTCATGTGTGGCGG	600 nM
	RdRp_SARSr P2	FAM CAGGTGGAACCTCATCAGGAGATGC BHQ	100 nM
	RdRp_SARSr R	CAAATGTTAAARACACTATTAGCATA	800 nM
CDC N1	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	500 nM
	2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC BHQ	125 nM
	2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG	500 nM

11. Jaeger *et al.*

Jaeger *et al.* characterize the primer dimers observed in these protocols and how these can create signals even with Taqman or probe hydrolysis based methods. This is a common complaint about our initial retraction letter. While we pointed out the primer dimer potential, most colleagues falsely assumed this was only a problem with SYBR green based qPCR. They are correct to point this out as SYBR green is much more prone to Primer-Dimer signals since its signal is derived from sequence-independent intercalating dyes. This non-specific amplicon labeling method usually requires a High Resolution Melt (HRM) analysis to confirm the target amplicon size. SYBR green based methods require this HRM step to confirm the specificity of the intercalating dye signal. Taqman or Hydrolysis probe based methods achieve this specificity by labelling a sequence-specific probe that is independent of the PCR primers. Jaeger *et al*. demonstrate probe hydrolysis can also occur as a result of primer dimers or primer-probe-background interactions in Taqman-based assays. Jaeger *et al.* even run gel electrophoresis on the samples with spurious qPCR signals and find primer dimers or other nonspecific signals. They cite Konrad and Pillonel as support for this.

"The apparent occurrence of dimerization does not appear to be exclusive to nucleocapsid targets. ***Unspecific signals in the late cycles of the envelope protein gene (E target) assay using the Charité protocol*** *(Konrad et al., 2020) and a mismatch of primer sequences (Pillonel et al., 2020) have been reported recently. The scientific community is discussing the technical limitations of the current SARS-CoV-2 RT-qPCR protocols (Marx, 2020) and their optimization is still underway."* [18] (Figure 19)

"*However,* ***fluorogenic probe-based reactions are not supposed*** *to be influenced by dimerization in the N2 primers–probe and/or primer–primer from the CDC RT-qPCR recommended protocol used for SARS-CoV-2 diagnosis. Won et al. (2020) found unspecific amplifications when using the N2 and N3 primers–probe sets and then proposed an alternative primers–probe panel for the nucleocapsid target*." [18] (Figure 19)

Note their specific comment that speaks of fluorogenic probe-based assays typically not generating signals but with these promiscuous primers they generate false positive signals**.**

Jaeger *et al.* also concludes:

"Finally, we recommend that RT-qPCR users adjust primers–probe and magnesium concentrations, the duration of the reverse transcriptase step, and the thermal cycle

temperature, independent of the master mix kit used, to minimize dimer formation and to avoid extensive test repetition and the waste of resources."

Figure 19 taken from Jaeger *et al.* (Figure 1):
Dimerization during RT-qPCR with the CDC N2 primers–probe set. Amplification plots of initial (A) and optimized (B) RT-qPCR conditions. Dimer formation can be visualized by the late signal produced in 'not detected' samples (curves 3, 4, and 5). Gel electrophoresis of initial (C) and optimized (D) RT-qPCR conditions. Dimers appear as diffuse bands (lanes 3, 4, 5) at the bottom of the gel (PCR products <50 bp). Partial sequence homologies between probe–probe (E), primer F–probe (F), and primer R–probe (G) estimated by OligoAnalyzer v.3.1. Key: 1 = no-template control (NTC); 2 = 2019-nCoV_N Positive Control (IDT); 3, 4, 5 = 'not detected' samples, 6, 7 = positive samples.

E
Delta G: -13.09 kcal/mole Base Pairs: 6
5' ACAATTTGCCCCCAGCGCTTCAG
3' GACTTCGCGACCCCCGTTTAACA

F
Delta G: -8.98 kcal/mole Base Pairs: 5
5' TTACAAACATTGGCCGCAAA
3' GACTTCGCGACCCCCGTTTAACA

G
Delta G: -9.89 kcal/mole Base Pairs: 4
5' GCGCGACATTCCGAAGAA
3' GACTTCGCGACCCCCGTTTAACA

12. Khan *et al.*

Khan *et al.* even discuss the propagation of an erroneous protocol having been circulated by the WHO and articulate the need to re-assess the suggested primers for SARS-CoV-2 RT-qPCR detection:

*"Despite the ability of single mismatches to be tolerated, it is important to consider that **mismatches need to be corrected** if found in most of the viral sequences available. For example, the reverse primer of Charité-ORF1b shows a mismatch with all the viral sequences (a total of 17 002). This mismatch has also been observed in 990 viral sequences along with the lower sensitivity of this assay in a recent preprint."*
[...]
"*However, some of the assays have not been reassessed in the light of the risk of mutations during viral evolution. Based on the analysis of 17 027 viral sequences, this study demonstrates the presence of mutations/mismatches in the primer/probe binding regions of some published assays (table 3). Sequences adjustments to these primers/probes need to be assessed experimentally using viral strains or nucleic acid coupled with subsequent experimental performance using clinical samples."* [19]

13. Opota *et al.*

Opota *et al*. [20] also abandon Charité's RdRp assays claiming:

"*Future studies should also include the comparison of in-house RdRP RT-PCR with commercial RT-PCR. Indeed, this comparison was not achieved as the RdRP RT-PCR needed further optimization based on recent publication that elucidated the reason of the limited sensitivity as the difference in the melting temperature of the forward and reverse primers of the initial PCR of Corman and colleagues (Corman and Drosten 2020; Muenchhoff et al. 2020; Pillonel et al.2020)."*
[...]
"*The RT-PCR targeting the RdRP gene and the N-gene were also introduced according to Corman and colleagues but showed a significantly reduced sensitivity **requiring further optimization and was not used for this comparison** (Pillonel et al. 2020)."*

14. Barra *et al*. (Preprint)

Barra *et al.* also make note of the reduced sensitivity of the RdRp assay. They test this against a modified RdRp assay and are careful to point out that the original Corman-Drosten primer set was never tested on real SARs-CoV-2 but on in-vitro transcribed SARs-CoV-2 RNA (IVT). In-vitro transcribed RNA does not contain the subgenomic RNA and therefore represents an ideal circumstance that isn't reflective of real world samples.

"The sensitivities observed in this study were slightly different than the described for RdRP (3.6 copies per reaction) and E (3.9 copies per reaction) original description, where the authors used the in vitro transcribed SARS-CoV-2 RNA directly in the reaction." [21], (Figure 20);

Figure 20 taken from Barra *et al.* (Preprint, Figure 2):
Assays limit of detection determination. N1 and RdRP (modified) showed better LOD. A) Raw data and B) Probit regression analysis (inserted unit values are copies/reaction).

15. Santos *et al.*

Santos *et al.* [22] aligned different primer / probe pairs against a broad collection of SARS CoV-2 gene sequences derived from Brazil. Here, they also report mismatches in the Charité's E primer sets:

"The French nCoV_IP4 and Chinese CN-CDC-E assays demonstrated total identity to their motives. The other assays, nCoV_IP2, CN-CDC-ORF1ab, Charité-E, and E_Sarbeco showed low frequency of errors, such as 1 to 2 bp mismatches."

"The assays 2019-nCoV (N1, N2, and N3), NIH-TH_N, nCoV_IP2, CN-CDC-ORF1ab, Charité-E, and E_Sarbeco, presented mismatches located in the 5' or central portion of their primers when aligned with the Brazilian viral genomes." [22] (Figure 21, Figure 22)

Figure 21 taken out of Santos *et al*. (Table 1):
List of analyzed assays by targets, frequency and location of mismatches. Each assay below includes three components, 2 primers and 1 probe. Both can be susceptible to matching errors.

Assays/Origen	Target	Total frequency of mismatches	Mismatches at 3' or 5' portion
US-CDC-N1/US-CDC	N	3/177	5' and 3'
US-CDC-N2/US-CDC	N	2/177	5'
US-CDC-N3/US-CDC	N	3/177	5' and 3'
NIID_2019-nCOV_N/Japan	N	0/177	-
N_Sarbeco/Japan	N	1/177	3'
CN-CDC-N/China	N	151/177	5'
HKU-N/Hong Kong	N	103/177	5' and 3'
NIH-TH_N/Thailand	N	2/177	5'
Corman-N/Germany	N	1/177	3'
nCoV_IP2/France	ORF1ab	1/177	5'
nCoV_IP4/France	ORF1ab	0/177	-
CN-CDC-ORF1ab/China	ORF1ab	2/177	5'
Charité-E/Germany	E	2/177	5'
CN-CDC-E/Germany	E	0/177	-
E_Sarbeco/France	E	2/177	5'

Note that the Chinese and Hong Kong assays for the N gene have many mismatches compared to the others. ORF1ab and E targets are less frequent in 3 'mismatches.

16. Anantharajah *et al.*

Anantharajah *et al.* described the evaluation of the primer/probe sets designed by the US CDC and Charité/Berlin (which is Corman et al) to detect clinical cases which were defined as “COVID-19 cases by chest CT”. In this work (Figure 22), the RdRp assay is once again the worst performing assay (lowest rate of positive detection, highest Ct value) amongst all tested, which was discussed to be based on the:

“Incorrect degenerate base S at position 12 that binds with G or C while all SARS-CoV-2 analyzed sequences encoded for a T at this position [...]. This mismatch would not be derived from a new variant but rather due to the initial oligonucleotide design allowing to amplify SARS-CoV, bat-SARS-related CoV and SARS-CoV-2-genomes.” [23]

"Among them, the United States Center for Disease Control (US CDC) recommended two nucleocapsid gene targets (N1 and N2) [3] while the German Consiliary Laboratory for Coronaviruses hosted at the Charité in Berlin (Charité/Berlin) recommended first line screening with the envelope (E) gene assay followed by a confirmatory assay using the RNA-dependent RNA polymerase (RdRp) gene, even before the first COVID–19 cases appeared in Europe. At the time of data submission 295 molecular assays are commercially available or in development for the diagnosis of COVID–19 and most of them use these recommended gene targets alone or in combination."
[...]
"*We observed notable mismatches in regions targeted by the primers/probe sets which might affect RT-qPCR assays performance depending on their location and the nature of the substitution."* [23]

The authors further discuss:

"The findings highlight substantial differences in sensitivity for the primer/probe sets when comparing under the same conditions. Indeed, N1 and N2 assays stand out in comparison with the E and RdRp assays for the detection of low-level viral loads. Furthermore, positive E and negative RdRp results were obtained in 15 cases. We may therefore question the need of confirmatory testing following an initial positive test according to the Charité/Berlin protocol, resulting in turnaround time delay and increased workload." [23]

Figure 22 taken from Anantharajah et al. (Figure 2):
Comparison of the viral load detected by the six RT-qPCR assays among the positive nasopharyngeal swabs (n = 84). The viral load is expressed in log copies/mL and each clinical sample is represented by a circle. The white circles represent clinical samples detected by all RT-qPCR assays while colored circles represent samples not detected by the six assays. Bars represent the median and 95% Condence Interval

17. Dahdouh *et al*

In a letter to the Editor of J. Infect., Dahdouh *et al.* highlight the Ct variance seen in the internal controls that target human DNA concurrent with SARS-CoV-2 detection (Figure 21).

As a conclusion, they point out:

"A full characterization of the linear ranges and a calibration using standards should be done for every different target and primer/probe design." [25]

The calibration and internal controls are missing completely in the Corman *et al.* PCR design.

Given the numerous examples presented of FP and FN generation with the quickly designed Corman-Drosten primers, there is a final intellectual challenge which this assay presents. Unlike most other SARs-CoV-2 qPCR assays, the Corman-Drosten assay lacks any internal control. The lack of such controls makes any measurement with the assay exposed to a significant source (4 logs) of variability as there is no reference to interpret the viral loads, which cannot be determined from Ct values without such reference to an internal control. Dahdouh *et al.* highlight the Ct variance seen in the Internal Controls that target human DNA concurrent with SARS-CoV-2 detection (Figure 23).

Figure in Dahdouh *et al.* demonstrates the Ct variance of Internal Control (IC Ct) on the Y axis compared to SARS-CoV-2 N gene Ct variance. Samples with high IC Ct represent poor patient sampling as too little human DNA is present to enable effective sample collection. The relative viral load can possibly only be estimated with reference to sampling efficiency, e.g. the IC signal.

Analysis of the SARS-CoV-2 Ct values obtained using a commercial RT-qPCR assay (Vircell) in a set of clinical samples. A) Cts of the Internal Control RNA plotted against the SARS-CoV-2 N gene Cts (r2 = 0.004).

Direct Link to Figure:
https://els-jbs-prod-cdn.jbs.elsevierhealth.com/cms/attachment/92b776fc-71d1-450e-9ede-1e08c9768393/gr1.jpg

This demonstrates that the patient sampling and DNA/RNA purification steps can alter the RNA/DNA yield 1,000-10,000 fold (10-13 Ct's). This is an important variance as the world debates 33 vs 37 Ct for calling patients infectious. If one can not measure sampling variance and normalize for this, one can't offer a rational Ct threshold upon which to classify a patient as infectious.

"The plot shows an inverse linear correlation, which is expected because Ct values reflect, indeed, viral loads, but the dispersion of the data may reach up to four log units (ten thousand-fold) for any given Ct (black arrow)." [25]

Normalizing for this 13 Ct variance cannot be done with the Corman-Drosten primer set as it does not contain a human genome target amplicon (RNaseP Internal Control). So not only does the protocol lack a description of which Viral Ct to call a positive, it doesn't have a human internal control to normalize for the 10,000 fold variance in nucleic acid sampling. This is very much frowned upon in clinical diagnostics. Incorporating human ICs requires

benchmarking to viral standards that are identical to the target virus (not distant relatives from bats or SARS). (Figure 24)

Figure 24: CDC guidelines for use of Internal controls from the RNase P gene. CDC: 2019-Novell Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. CDC-006-00019, Revision 05. 13.07.2020

2019 nCoV_N1	2019 nCoV_N2	RP	Result Interpretation[a]	Report	Actions
+	+	±	2019-nCoV detected	Positive 2019-nCoV	Report results to CDC and sender.
If only one of the two targets is positive		±	Inconclusive Result	Inconclusive	Repeat testing of nucleic acid and/or re-extract and repeat rRT-PCR. If the repeated result remains inconclusive, contact your State Public Health Laboratory or CDC for instructions for transfer of the specimen or further guidance.
-	-	+	2019-nCoV not detected	Not Detected	Report results to sender. Consider testing for other respiratory viruses.[b]
-	-	-	Invalid Result	Invalid	Repeat extraction and rRT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

[a]Laboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.
[b]Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest that 2019-nCoV infection is possible, and diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If 2019-nCoV infection is still suspected, re-testing should be considered in consultation with public health authorities.

RNase P (Extraction Control)

- All clinical samples should exhibit fluorescence growth curves in the RNase P reaction that cross the threshold line within 40.00 cycles (< 40.00 Ct), thus indicating the presence of the human RNase P gene. Failure to detect RNase P in any clinical specimens may indicate:
  - Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
  - Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
  - Improper assay set up and execution.
  - Reagent or equipment malfunction.
- If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:
  - If the 2019-nCoV N1 and N2are positive even in the absence of a positive RP, the result should be considered valid. It is possible, that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RP signal does not preclude the presence of 2019-nCoV virus RNA in a clinical specimen.
  - If all 2019-nCoV markers AND RNase P are negative for the specimen, the result should be considered invalid for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.

18. Poljak *et al.*

The RdRp (RNA-dependent RNA polymerase) gene is a synonymous nomenclature. This enzyme is encoded by the nsp12 gene which is part of ORF1. RdRp is the cleavage product of the polyproteins 1a and 1ab from ORF1a and ORF1ab [43,53]. There is a high degree of conservation among RNA-dependent RNA polymerases of different RNA viruses which explains its lack of specificity to SARS-CoV-2.

Roche replaces the RdRp Corman primer with a more specific primer pair for SARS-CoV-2 called ORF1, also includes an Internal Control to monitor the sample preparation variance and also implements an enzymatic decontamination process (UDG) to reduce false positives. Four false positives are evident in the original Corman paper. The authors justify these false positives as 'user error' but since they lack the correct controls, this cannot be discerned from the information published and is a false conclusion derived from the data provided.

"The test utilizes RNA internal control for sample preparation and PCR 167 amplification process control. Uracil-N-glycosylase is included in the PCR mix to destroy 168 potential contaminating amplicons from previous PCR runs." [40]

The last paragraph of the results section states:

"*After extensive evaluation, our laboratory implemented LightMix-based SARS-CoV-2 testing on 17 January 2020."* [40]

This manuscript also sheds light on the timelines of disclosure for this test. Slovenia already had the TIB Molbiol LightMix earlier than January 17 2020, a period when no case of the "new virus" was even documented in Europe. Further, we can also conclude that TIB Molbiol (Olfert Landt) distributed those PCR kits with the Corman Drosten primers and probes at least one week before they submitted the original manuscript describing the protocol-design to Eurosurveillance, and presumably in parallel they were also sending out the protocol to the WHO.

Summary of the Poljak Methods:

- A) LightMix Modular SARS and Wuhan CoV E-gene kit and RdRp gene kit were used, the protocol followed the CormanDrsoten protocol, Ct values above 37 were considered negative.
- B) Cobas 6800 SARS-CoV-2 testing for the ORF1 gene and the Sarbeco E gene

Results:
First test (in-house panel):
2 of 217 samples were excluded from analysis due to invalid cobas results;
3 of 63 samples which were positive with the LightMix were negative by cobas;
1 of 152 samples which were negative by LightMix were found positive by cobas;

211/215 results were identical;

Second test (prospective comparison).
1 of 502 samples was excluded from analysis due to invalid cobas results
2 of 438 samples which were negative by LightMix were found positive by cobas

A correlation is shown in Fig 1 (Figure 25) for the positive samples in relation to the CT values of the RdRp gene and the ORF-1 gene.

Of note: Fig 1 in Poljak *et al.* does not show the correlation for the E-Gene, neither is this crucial data shown in the results or in the discussion section: the relevant data here is simply missing. About 28 samples had a Ct higher than 35 for the ORF-1/RdRp gene

Figure 25 taken from Poljak et al. (FIG 1):

FIG 1: Correlation between cycle threshold (Ct) values obtained by LightMix Modular Wuhan 406 CoV (RdRP gene – SARS-CoV-2 specific target) and cobas 6800 SARS-CoV-2 (target 1 – ORF1 – 407 SARS-CoV-2 specific target) in the prospective head-to-head evaluation performed on 502 408 samples. Ct values for the LightMix assay were always set to 0.1 normalized reporter dye 409 intensity (delta Rn). Linear regression of the Ct values was performed using samples positive for 410 SARS-CoV-2 by both diagnostic approaches (n = 63). The r² correlation value is indicated.

19. Boutin *et al.*

The authors compared an in-house test following the E-gene primers and probes of the Corman Drosten-protocol according to their publication with the Roché Cobas SARS-CoV-2 test, spanning the targets ORF1 and PAN-Sarbeco E gene [41].

Methods:

Additionally the Abbot real time SARS-CoV-2 test was used to clear discrepant results. Detection limit was quoted as 180 viral RNA copies per ml with the in-house test and 23 viral RNA copies per ml the Roché Cobas test.

Samples: 377 routinely collected nasopharyngeal/oropharyngeal swabs.

121 of those: no symptoms
132 symptomatic (no further definition is given on what type of symptoms)
124 without information

Results:

In-house E-gene: 281 of 377 samples were found positive ("detected") and 96 negative ("non detected"). This means a rate of positive samples of 74% .

Cobas: 301 samples were found positive and 76 negative (rate of positive samples: 80%);

Note: since at least 124 of the 377 samples were from asymptomatic patients, the rate of positivity is remarkably high.

Even if all individuals for whom no information is available were symptomatic, in total 256 symptomatic persons (68% of all) were tested, which means that from the defined asymptomatic persons, 25 (equals 21% with the in-house test) or 45 (equals 37% with the Cobas test) were found to be positive.

Concordance of the test results:

22 of the samples which were positive in the Cobas test were negative with the in-house Corman-Drosten E gene assay. Two samples were negative in the Cobas test for both gene targets but were positive for the Corman-Drosten E-gene test. 74 of 88 samples were tested negative with both tests (negative agreement 84,1%).

All discordant samples had high Ct-values (35 or higher). The majority of positive samples in the Cobas had CTs of 30-39.

Boutin et al. (Figure 1): from Boutin *et al.* demonstrates high concordance at low Ct with less concordance at high Ct.

Correlation between cycle thresholds (Ct) values obtained with the cobas 8800 SARS-CoV-2 assay for target -1 Orf1 gene and target 2 –E gene (pan-sabercovirus detection) in 279 positive samples for SARS-CoV-2 virus RNA. The dotted line is the 95 % confidence internal of the regression line.

Direct-link to Boutin et al. (Figure 1):
https://pubmed.ncbi.nlm.nih.gov/32927356/#&gid=article-figures&pid=fig-1-uid-0

Re-testing of 20 of the 22 samples that were positive with Cobas but negative at the Charité E-gene, the Abbot system resulted in 8 “detected”, 11 “non detected” and one impossible result.

Re-test of the 11 negative samples with the Abbot test (initially positive with the Cobas test) revealed one positive result in the re-test with the Coabs system and 10 negative results. According to the authors this result was due to limited storage possibility of the samples.

Boutin Discussion:

The authors claim that there is currently no gold standard for the diagnosis of SARS-CoV-2 infection. Limit of detection was now given with 300 SARS-CoV-2 RNA copies per ml sample (was 180 in the Materials section). Despite the difference in the detected samples (negative agreement only 84.1%, so 15.9% difference), the authors conclude that their study demonstrates an excellent agreement between the Cobas Sars-CoV-2 test and the in-house Sarbeco E (Drosten-Corman Test).

Evaluation: the study clearly shows:

- The test system used for PCR defines the type of positive findings (here 68% vs. 80%) with a remarkable high difference (15%) with different tests applied to the same samples.
- The majority of positive samples with both test systems were found at a Ct higher than 30 or even 35.
- Since the findings were not assigned to the symptomatic/asymptomatic/unknown clinical data of patients, no correlation of result against Ct with clinical data is possible.

20. Pfefferle *et al.*

Pfefferle *et al.* used the original Corman E-primer pair and probes, but: ”Both primers were modified with 2’-O-methyl bases in their **penultimate base to prevent formation of primer dimers.“** They did not test the PCR on patient samples, but on *in vitro* transcribed E-Gene RNA of SARS-CoV-2 only. So the authors of this very early publication (submitted Feb 14, 2020) pointed out that the original Drosten/Corman E-gene PCR primers were prone to primer dimers and that the PCR should always be confirmed with a second independent PCR.

The authors note:

"It has to be noted that by its nature as a screening test targeting only a single viral gene, positive results should always be confirmed with an independent PCR as recommended." [42]

b. Summary wet-lab evidence of primer design flaws

In summary, the peer-reviewed literature on the defects of the Corman-Drosten primers is vast. While biases and errors may be understandable due to pandemic time constraints, those due to short-circuited peer review, conflicts of interest and regulatory capture at the WHO, should be condemned once they are identified. There is no way to maintain public trust in the scientific method and publication process when such errors affect millions of people's clinical decisions and livelihoods.

This is no subtle oversight as it is well established in clinical diagnostics that internal controls and Ct correlations with replication competent organisms are a requirement to benchmark any Ct score to biological meaning. Many papers now describe how to properly perform such calibrations with PFU and Ct scores like Jaafar *et al.* [26]. Some even describe more comprehensive methods to understand infectious vs non-infectious patients with careful attention to subgenomic RNA and genomic RNA (Wölfel *et al.* [37] and Liotti *et al.* [27]).

Walker *et al.* [28] even demonstrate that only 72% of the samples produce positive results when 3 gene targets are utilized. 28% of samples only amplify with 1 or 2 of the assays failing, suggestive of degraded and non-infectious RNA due to an amplicon design focus on targeting the highly expressed 3 prime subgenomic RNAs (Figure 24). Assays cutting corners and relying solely on 1 or 2 assays (targeting non-replication competent subgenomic RNA) with no internal controls create erroneous results and quarantines an excessive number of non-infectious people.

Figure 26: Table reproduced from Walker *et al.* demonstrating the number of positive tests where 1,2 and 3 genes amplify.

Table 1 Genes detected in positive swabs

	All positives (N=1892)		First positive per participant (N=1516)	
Number of genes detected	n (%)	Median CT* (IQR) [range]	n (%)	Median CT* (IQR) [range]
1	345 (18%)	33.6 (32.3-34.6) [12.7-37.6]	307 (20%)	33.7 (32.5-34.7) [12.7-37.3]
2	185 (10%)	31.5 (29.8-32.8) [10.3-36.3]	138 (9%)	31.5 (29.6-33.0) [10.3-36.3]
3	1362 (72%)	22.8 (18.2-27.4) [10.5-34.2]	1071 (71%)	21.8 (17.7-27.0) [10.5-33.8]
Genes detected				
N only	243 (13%)	33.7 (32.5-34.7) [29.0-37.6]	213 (14%)	33.8 (32.6-34.7) [29.0-37.1]
ORF1ab only	83 (4%)	32.7 (31.9-33.8) [24.0-35.7]	75 (5%)	33.0 (31.9-33.9) [24.0-35.7]
S only**	19 (1%)	35.0 (34.3-36.1) [12.7-37.3]	19 (1%)	35.0 (34.3-36.1) [12.7-37.3]
N+ORF1ab	158 (8%)	31.3 (29.8-32.6) [10.3-36.3]	113 (7%)	31.2 (29.6-32.8) [10.3-36.3]
S+ORF1ab	9 (0.5%)	28.9 (26.1-31.0) [16.2-34.7]	8 (0.5%)	28.8 (24.5-32.1) [16.2-34.7]
N+S	18 (1%)	32.8 (32.3-33.1) [28.2-35.2]	17 (1%)	32.8 (32.3-33.1) [28.2-35.2]
N+S+ORF1ab	1362 (72%)	22.8 (18.2-27.4) [10.5-34.2]	1071 (71%)	21.8 (17.7-27.0) [10.5-33.8]

* taking the mean CT per positive swab across positive gene targets (Spearman rho=0.99 for each pair of genes, $p<0.0001$)
** through mid-May only: after this samples positive for the S gene only were not called positive overall.
Note: comparing first vs subsequent positives per participant, exact $p<0.0001$ for both number of genes detected and specific genes detected.

Liotti *et al.* [27] demonstrate qPCR positivity can last as long as 77 days (48.6 mean) past symptom development but only observe 7-10 days of infectiousness (n=176) when benchmarking the Ct values against cell culture. Increasing the sample size of a study like Liotti *et al*. is likely to find cases that extend the long tail of qPCR positivity post-recovery and post-infectiousness. Liotti *et al.* implies the vast majority of qPCR positive samples will be non-infectious patients. They describe a mean of 48.6 days of qPCR positive. They also describe 7-10 days of infectiousness. This produces a range of non-infectious qPCR positive to infectious qPCR positive ratio of 4.86:1 to 11:1. This is an alarming rate of quarantine for non-infectious patients. To quarantine a patient, you must have evidence of existing infectiousness, not RNA from a past infection. The Corman-Drosten manuscript ignores this medical ethics question whilst also compromising the accelerated peer-review process by a gross failure to disclose financial conflicts of interest.

The authors' premature escalation of their work to the WHO prior to peer review is alarming. The lab testing revenue and therefore conflicts of interest of various authors were not properly disclosed in the initial Eurosurveillance peer review. Had the journal been aware of the conflicts they may have placed more scrutiny on the review.

Likewise, we have not seen the authors exhibit the same urgency in updating the WHO regarding the reported false positives from the hastily reviewed Corman-Drosten paper. This raises important questions regarding the lab testing conflicts of interest of various authors.

Increased qPCR positivity amplifies testing revenue through follow-on track-and-trace testing revenue. This places public health and citizen freedom in direct conflict with heavily funded testing labs who clearly have financial interests in higher test positivity.

Section 2:

B. Meta-data Analysis on EuroSurveillance.org (peer review timeframes)

Additional work was provided profiling the peer-review timeframes at Eurosurveillance by Wouter Aukema, who has over 30 years of experience in processing and analysing data for governments and corporations world-wide and develops data analysis solutions for Fortune 100 companies. His publication at Defcon (20 years ago) caused headlines worldwide as it identified significant software virus vulnerabilities to Lotus Notes [29].

This analysis by Wouter Aukema provides additional evidence of the exceptional short review time for a manuscript that, at the time, didn't fully disclose the authors' conflicts of interests. This puts the journal in a very compromised position as it may have been scrutinised more had the conflicts been disclosed during the rushed review. Instead these conflicts were brought to light after the rushed review and publication.

The goal is to understand how much time it typically takes for research papers to get reviewed and accepted by eurosurveillance.org. [29], (Figure 27);

The reason for this assessment is to provide clarity around discussions of a specific research paper that was reviewed and accepted in a single day. Some scientists think it is impossible to Peer-Review research within a single day. Other scientists claim the paper went through the much quicker- Rapid Review procedure, as outlined on the journal's web site.

To assess commonality in the review and acceptance process at eurosurveillance.org, the author collected and analysed meta-data for all 1,595 publications since 01-Jan-2015. Earlier this week, the author shared the initial findings of this assessment in a Twitter post.

This six-page document aims to make these findings reproducible and verifiable by offering step by step instructions.

Summary of Findings:

- *Of the 17 types of articles published since 2015, three types occur most frequently: Rapid Communication (385), Research (312) and Surveillance (193).*

- *The average number of days between Acceptance and Reception of Research type articles is 172 (2019) and 97 (2020).*
- *In line with the Editorial Policy for Authors, the category 'Rapid Communication' publications appear to be reviewed and accepted more quickly (18 days average) than type 'Research' and 'Surveillance.'*
- *Except for this one Research article (on 22-jan-2020), no other article has ever been reviewed and accepted within a single day since 2015.*

Figure 27: Dot plot of peer review timelines for manuscripts published at Eurosurveillance since 2015. The Corman-Drosten paper is an extreme outlier.

The corresponding author (in this case Christian Drosten) had to fill out a section called "Agreement with authors" at the Eurosurveillance Submission portal, a mandatory requirement and document for successful submission. Christian Drosten had to confirm that there were no conflicts of interests. We can clearly conclude that he was not honest while filling out the form back in January 2020. Six months later into the pandemic an Update was added for Marco Kaiser under the section "conflicts of interests", who is senior researcher at

GenExpress and serves as scientific advisor for Tib-Molbiol. Given the unbelievably short review time, we have to further conclude:

- The editor in charge found experts that are willing to review within hours.
- All experts immediately reviewed the manuscript and declared it as perfect, as it is.
- The editor immediately handled the review reports.

Nevertheless, after acceptance the paper still needs to be sent to a typesetter, even though it had immediately received the “Accept” status without any major or minor revisions.

The timeline of the Corman-Drosten Peer Review demonstrates digital timestamps on documents sent to the WHO at 20:30 CET on Jan 21-2020. The paper submitted to Eurosurveillance on the same day references the WHO document and is assumed to have been submitted after 20:30 CET as it's impossible to reference a WHO document unless the WHO document was submitted first. This leaves 3.5hrs to 27.5 hrs for review as the paper was accepted the next day on Jan 22, 2020. Given the late evening submission, reviewers would have to be recruited off-hours, agree to review the paper and complete the review mostly outside of business hours. (Figure 28)

Figure 28: Possible review time estimation Corman Drosten et al.

C. Missing positive controls for PCR test validation

This chapter further investigates the positive controls referenced in Muenchhoff *et al*, Mautheeussen *et al.* and Wolf *et al.*

The positive controls used to prepare the RNA dilution series as the basis for the Corman RT-PCR-testing were described as a sample deriving from a five-year-old child with COVID-19. As source, Wolf *et al.* is cited. The methods section states:

"*Nasopharyngeal swabs were used for virus culture in a biosafety level 3 laboratory on Vero cells.*" [6]

The results section of the Wolf *et al*. paper which is referenced in the Muenchhoff *et al*. paper further concludes:

"*She did not develop any respiratory symptoms but tested PCR-positive again in nasal and pharyngeal swabs on 3rd February when infectious viruses could be grown from swab material."* [6]

According to Wolf *et al.,* a pathogen was isolated and cultured from the patient. Further, for her two-year old brother, they noticed:

"As with his sister, the infectious virus was easily grown from the nasopharyngeal swab material on 3rd and 4th February." [6]

Following these statements there should have been two virus isolates available for the Muenchhoff *et al.* study (submitted 28th May 2020), but they didn't characterize the RNA isolated from the samples. The Matheeussen *et al.* publication [33] (submitted two weeks later), claimed that SARS-CoV-2 isolates are used as a source for the positive control RNA.

Neither the Wolf *et al.* publication, nor the Muenchhoff *et al.* or Matheeussen *et al.* describe how the virus isolates / RNA used in the assay validation is characterized. There is no data available concerning sequence validation of these targets and no information about the virus in general.

Wolf *et al.* and Muenchhoff *et al.* list the Institute for Virology in Munich as the main research-hub / institution & correspondence. Christian Drosten is co-author of the Muenchhoff *et al.* & Mautheussen *et al.* publications. Victor Corman is the second author of the latter paper. The audit trail for the "true positive" controls used for the basis of the test is thus incomplete. This makes it impossible for labs to directly replicate the work.

In a recent Lancet publication Surkova *et al*.[46] it is stated:

"RT-PCR tests to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA are the operational gold standard for detecting COVID-19 disease in clinical practice.
[...], but no single gold standard assay exists." [46]

In other words, the sensitivity and specificity of PCR are determined with the PCR test itself as "operational gold standard". PCR tests should be calibrated to replication competent organisms. Use of PCR to validate PCR is circular reasoning.

Surkova *et al.* references a British Medical Journal article, Watson *et al*., and there we can find following further conclusions:

"*No test gives a 100% accurate result; tests need to be evaluated to determine their sensitivity and specificity, ideally by comparison with a "gold standard." The lack of such a clear-cut "gold-standard" for covid-19 testing makes evaluation of test accuracy challenging.*
A systematic review of the accuracy of covid-19 tests reported false negative rates of between 2% and 29% (equating to sensitivity of 71-98%), based on negative RT-PCR tests which were positive on repeat testing. The use of repeat RT-PCR testing as gold standard is likely to underestimate the true rate of false negatives, as not all patients in the included studies received repeat testing and those with clinically diagnosed covid-19 were not considered as actually having covid-19." [46]

D. *In silico* Analysis, Primer homology to human DNA

We have performed additional analysis to address concerns voiced regarding the Charité primers and their homology to human DNA.

We have included a BLAST analysis of the Charité primers against the Human Genome (GRCh38.p13). There are several significant homologies but none that have both primer and probes in close proximity. While these off-target homologies are not catastrophic for assay performance, they do demonstrate the lack of *in silico* analysis done prior to publication and they may play a role in the in-vitro synthesis of more diverse 3 prime ends of primers during the cold (55C) reverse transcription step of RT-qPCR. The BLAST output file is available for download in the references section [30]. With the shortage of RNA purification kits in 2020, many labs are using modified purification protocols that omit the DNAse step thus leaving human DNA as a viable target of primers (Figure 28) [32].

Wozniak et al. describe a more automatable and streamlined RNA preparation for SARs-CoV-2 qPCR. They omit the DNase step to reduce consumables and notice it benefits their internal control signal. The authors conclude:

"DNase treatment is not necessary because SARS-CoV-2 detection is not altered in the presence of DNA. In fact, residual DNA may serve as the template for RNase P gene amplification."

Figure 29 shows the 18bp 3 prime homology found in the RdRp Reverse primer to human chromosome 18.

Figure 29: BLAST alignment using blastdb -task blastn-short -query Corman_Primers.fa -db GRCh38.p13.fna. Query is the RdRp Reverse primer and Sbjct = Human Genome reference genome GRCh38.p13 Primary Assembly in NCBI.

```
> NC_000018.10 Homo sapiens chromosome 18, GRCh38.p13 Primary Assembly
Length=80373285

 Score = 36.2 bits (18),  Expect = 0.34
 Identities = 18/18 (100%), Gaps = 0/18 (0%)
 Strand=Plus/Plus

Query  9         AAAGACACTATTAGCATA  26
                 ||||||||||||||||||
Sbjct  45238971  AAAGACACTATTAGCATA  45238988
```

E. Further Discussion - The Consequences of False Positives / False Negatives

We further conclude that the origin of the problem is not solely technical in nature but also not fit for the intended clinical purpose in the Corman Drosten-paper.

We aimed to develop and deploy robust diagnostic methodology for use in public health laboratory settings without having virus material available. [44]

This misguided aim is already discussed in the main review report Pieter Borger *et al*. [4] in great detail, nevertheless we see the need to re-emphasize the misguided premise at this point and to extend our critique on population mass-testing through the means of RT-qPCR.

Even if the RT-qPCR test was optimal and had theoretically sensitivity and specificity of 100%, it is medical malpractice to use RT-qPCR and other rapid tests outside the need for specific antiviral therapy in symptomatic or severely ill hospitalised patients. Interpreting

positive tests as 'medical cases' without consideration of internal controls and viral Ct with clinical context, nor consideration of other viruses or diseases that cause similar symptoms as COVID-19, enables politicians to practice medicine on entire populations. This lack of clinical integration has led to problems in the past.

Blind faith in a quick RT-qPCR-test has created a pseudo-epidemic described in this New York Times article in 2007 [34]:

"I had a feeling at the time that this gave us a shadow of a hint of what it might be like during a pandemic flu epidemic.
[...]
Yet, epidemiologists say, one of the most troubling aspects of the pseudo-epidemic is that all the decisions seemed so sensible at the time."

Even Christian Drosten admitted himself in a German Article in 2014 the very problem of RT-qPCR tests in a pandemic or epidemic scenario:

"The method is so sensitive that it can detect a single genetic molecule of the virus. If, for example, such a pathogen flies over the nasal mucous membrane of a nurse for a day without them becoming ill or noticing anything, then it is suddenly a MERS case. Where previously terminally ill were reported, now suddenly mild cases and people who are actually very healthy are included in the reporting statistics. This could also explain the explosion in the number of cases in Saudi Arabia." [45]

Furthermore, the WHO falsely claims in an official document:

"In areas where COVID-19 virus is widely spread a simpler algorithm might be adopted in which, for example, screening by rRT-PCR of a single discriminatory target is considered sufficient." [48]

A single confirmatory gene assay can never be sufficient enough for accurate testing-results, especially not in a mass-testing scenario. [42]

The PCR testing with the E-gene (Corman-Drosten *et al.*) is also used in single-gene PCR tests in the EU and has been demonstrated to be unspecific for the detection of SARS-CoV-2 [49].

“A high amount of specificity means, that the test is able to detect SARS-CoV-2 infections, only. In contrast, PCR tests with a rather lower specificity might pick up all kinds of other Corona viruses. The lower the specificity, the lower the ability to prove the infection by a specific virus.” [47]

This is an important point to underscore. According to Corman *et al*. they describe their RdRp gene as having low specificity yet this is a confirmatory assay that has many design flaws and documented deficiencies in the literature.

"Detection of these phylogenetic outliers within the SARS-related CoV clade suggests that all Asian viruses are likely to be detected." [44]

The E-gene also has documented deficiencies and the test has no internal controls or calibration to replication competent organisms or PFUs. The genbank accession numbers in NCBI do not contain any E gene sequences to demonstrate the assay is functional.

The Corman Drosten-protocol results can not be reproduced.

The consequences of false-positives are further discussed in an article by Howard Steen & Saji Homeed [35] and in an article by Michael Yeadon, titled *The PCR False Positive Pseudo-Epidemic* [36].

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